Cleavage of immunoglobulin A1, A2 and G by proteases from clinical isolates of *Pasteurella multocida*

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Summary. Several *Pasteurella multocida* strains were examined for their ability to produce extracellular enzymes that cleave immunoglobulin A and G (Ig A and Ig G) molecules. Two strains isolated from human pulmonary and genital infections produced proteases that cleaved human IgA and IgG, colostral IgA and human myeloma IgA1 and IgA2. Human IgM was not degraded by these enzymes. Examination of cleavage digests showed two main fragments with different electrophoretic mobilities. The two *P. multocida* strains produced a protease that cleaved IgA and IgG heavy chains outside the hinge region, and differed in this respect from the hinge-cutting proteases of other bacteria. Protease production may be a virulence mechanism for *P. multocida* strains.

Introduction

The primary functions of immunoglobulin A (IgA) are to protect the mucosae and underlying tissue from bacterial invasion and to neutralise toxins.^{1,2} To remain fully functional, IgA molecules must maintain their integrity within the hostile enzymatic environment of the mucosae. However, they are attacked by the proteases of pathogenic bacteria such as *Neisseria gonorrhoeae*, *N. meningitidis*, *Haemophilus influenzae* and *Streptococcus pneumoniae*.^{3–5} These proteases are remarkable in their specificity for IgA1, with a cleavage site localised in the hinge region within a short peptide sequence which is deleted in IgA2.⁶

More recently, IgA protease synthesis was detected in bacteria freshly isolated from urinary tract infections,⁷ but this property was readily lost on subculture. Some Enterobacteriaceae, *Proteus* spp. and *Serratia marcescens* are now known to produce IgA proteases.^{8,9} The capacity to produce such enzymes appears to be a common characteristic of bacteria that frequently colonise the mucosae.

Pasteurella multocida is a major pathogen of animals and has been associated increasingly with human infections. Soft tissue infections resulting from animal bites or scratches are predominant.¹⁰ However, *P. multocida* is also capable of colonising respiratory or genital mucosae.^{10–12} The production of IgA protease by laboratory strains of *P. multocida* could not be demonstrated.¹³ This study examined its production by strains freshly isolated from man.

Materials and methods

P. multocida strains

The three strains examined were of human origin. Strain PMR 12 was isolated from a sinus specimen, PMR 25 from a coil specimen, and PMR 40 from the sputum of a patient with pleuropneumopathy. The strains were identified by standard criteria.¹⁴ Tests for acid production from carbohydrates gave results that matched those of the subspecies *multocida* described by Mutters *et al.*¹⁵ The three strains were of the D capsular type, as determined by the acriflavine test.¹⁶ Protease production was investigated after less than three subcultures.

Immunoglobulin preparations

Serum and secretory IgA were purified with a lectin (jacalin). A jacalin crude extract (JCE) was prepared as described previously.¹⁷ Purified jacalin was obtained from the JCE by affinity chromatography on agarose-amino-caproyl-D-galactosamine (Aggalactosamine; Pharmacia, Uppsala, Sweden). Rehydrated gel (5 ml) was equilibrated with 0.5 M NaCl and 0.01 M phosphate buffer at pH 7.2. JCE (30 mg) was incubated with the gel for 2 h at room temperature. After washing with buffer, the fixed material was eluted with 0.1 M glycine buffer at pH 3.5, immediately neutralised with 1 M Tris, and dialysed against 0.01 M phosphate-buffered saline (PBS).

Polyclonal monomeric IgA was purified from a normal human serum pool by means of the jacalin column, as described by Andre *et al.*¹⁸ Serum (5 ml) was applied to the jacalin column equilibrated in 0.5 M NaCl and 0.1 M phosphate buffer at pH 7.4. After

incubation for 1 h at room temperature, the column was thoroughly washed with the same buffer until zero optical density (OD) was detected at 280 nm. The bound proteins were eluted with a Tris-glycine buffer, pH 3.0, and immediately buffered and dialysed against PBS. IgA was then separated by gel filtration on a calibrated Sephacryl S 300 column (Pharmacia). Polymeric secretory IgA from skimmed colostrum was purified according to the method of Roque-Barreira and Campos-Neto,¹⁹ after precipitation of casein in acid medium.

Purified myeloma IgAs, kindly provided by Dr Aucouturier, Poitiers, France, were as follows: Cog IgA1 L, Per IgA1 L, Bel IgA2m(2) K, Felg IgA2m(2) L and Gir IgA2m(1) K.

IgG was purified by affinity chromatography on a sepharose 4B-protein A column from a pool of donor sera, following the manufacturer's recommendations (Pharmacia). IgM was purchased from Tago (Burlingame, CA, USA).

Preparation of crude microbial proteases

The procedure for obtaining crude preparations of these enzymes was adapted from the method of Higerd *et al.*²⁰ Briefly, sterile dialysis tubing on the surface of cooked blood agar was inoculated by a swab with overnight cultures (37 °C) of *P. multocida* from cooked blood agar. The plates were incubated overnight at 37 °C aerobically. The dialysis tubing was removed and thoroughly washed with a minimal volume of PBS. After centrifugation to remove the bacteria, the supernate containing extracellular protease was removed and concentrated approximately 20-fold by applying negative pressure through a dialysis membrane (Micro-Prodicon, Beaverton, OR, USA) against PBS. Protein concentrations were determined by a dye-binding assay (BioRad, Richmond, CA, USA). Preparations were stored at -80 °C until used.

Detection of protease activity

The various immunoglobulin and protease preparations (20 µl of 700 µg/ml solution) were mixed and incubated for 18 h at 37 °C. The enzyme preparation was replaced by buffer in control samples. Proteolysis was detected by gel electrophoresis and immunoblotting. Acrylamide 3 and 10% stacking and separating gels (LKB Instruments Inc., Rockville, MD, USA) were prepared for SDS-PAGE. Enzymatic digestion products were diluted 50% in a buffer solution containing, at final concentration, sodium dodecyl sulphate 2% w/v, β-mercaptoethanol 5% v/v, glycerol 10% v/v, bromophenol blue 0.1% w/v, and 0.1 M Tris hydrochloride (pH 6.8). The samples were boiled for 2 min and then a 30-µl volume was added to each well. Low mol. wt markers (5µl, Pharmacia) were boiled for 2 min and run in parallel. Electrophoresis was performed in a vertical electrophoresis unit (2001 model, LKB) at 60 mA for 15 min

for the samples to penetrate the gels, and then at a constant 100 mA current until migration was complete (2–3 h). The gels were then transferred to a nitrocellulose membrane, pore diameter 0.45 µm (BioRad).

Immunoblotting was done as described by Towbin *et al.*²¹ Transfer took 2 h at 0.5 mA and 4 °C in a 2005 model Transphor unit (LKB). The nitrocellulose was blocked for 16 h at 4 °C in PBS containing bovine serum albumin 0.1% and Tween 20 0.05%. The nitrocellulose was incubated for 1.5 h at room temperature with one of the following goat anti-human F(ab')₂: IgA, IgG or IgM, combined with peroxidase (Tago). After three washes with PBS-Tween 20, 3,3'-diaminobenzidine tetrahydrochloride (Prolabo, Paris, France) 0.5 mg/ml in 0.1 M Tris hydrochloride (pH 7.6)-hydrogen peroxide 0.1% was added and the reaction was stopped with an acetic acid 0.5% solution as soon as the bands became visible.

Results

Detection of protease activity on serum IgA (fig. 1)

Incubation with the preparation from strain PMR 12 did not result in the cleavage of serum IgA into different fragments. Conversely, with preparations from strains PMR 25 and PMR 40, IgA degradation products were visible. Two main fragments were observed after treatment with the protease preparation from strain PMR 40, with apparent *M_r* of 44 and 37 kDa, in contrast with 61 kDa for the heavy chain α. One main fragment (44 kDa) was seen with the protease preparation from strain PMR 25. Strain PMR 40 protease appeared to have greater activity, resulting in the almost total disappearance of the IgA band; a slight 2–3 kDa shift in the IgA band, similar to that observed with the strain PMR 12 preparation, was also found.

Detection of protease activity on secretory IgA (fig. 2)

Like serum IgA, secretory IgA was not cleaved by the preparation from strain PMR 12. However, protease preparations from strains PMR 25 and PMR 40 almost completely degraded colostral IgA. Two fragments (44 and 36 kDa) were seen with the strain PMR 40 preparation, and one fragment (44 kDa) with the strain PMR 25 preparation. Here again, the protease preparation from strain PMR 40 appeared to be more active than that from strain PMR 25.

Detection of protease activity on myelomatous IgA (fig. 3)

The preparation from strain PMR 40 was then used to study the action of proteases on IgA myelomas corresponding to the two IgA isotypes and the two IgA2 allotypes. With the heavy chain of the Per IgA1

Fig. 1. Immunoblot of serum IgA before and after digestion by crude protease preparations from *P. multocida*. Lanes 1, 3, 5, serum IgA control; serum IgA after incubation with preparations from strains PMR 12 (lane 2), PMR 25 (4), and PMR 40 (6).

Fig. 2. Immunoblot of secretory IgA. Lanes 1, 3, 5, secretory IgA control; secretory IgA after incubation with preparations from strains PMR 12 (lane 2), PMR 25 (4) and PMR 40 (6).

Fig. 3. Immunoblot of myelomatous IgA before and after digestion by crude protease preparations from strain PMR 40. Lane 1, Per IgA1; 2, Per IgA1 with PMR 40; 3, Cog IgA1; 4, Cog IgA1 with PMR 40; 5, Felg IgA2m(2); 6, Felg IgA2m(2) with PMR 40; 7, Bel IgA2m(2); 8, Bel IgA2m(2) with PMR 40; 9, Gir IgA2m(1); 10, Gir IgA2m(1) with PMR 40.

(60 kDa), two fragments (44 and 37 kDa) were visible; and with the heavy chain of the Cog IgA1 (62 kDa), two fragments (46 and 39 kDa) were also found.

With the two IgA2m(2) myelomas, Bel and Felg, there were two main cleavage fragments. They were obtained in similar proportions compared to the heavy chains (63 kDa for Bel and 59 kDa for Felg): 45 and 39 kDa for Bel, and 41 and 35 kDa for Felg. There was a third migration fragment, slightly faster than the heavy chain.

With the Gir IgA2m(1) myeloma, the intact heavy chain had an apparent M_r of 63 kDa, and bands of 44 and 36 kDa were produced. The initial rapid migration band was not found with this allotype.

Detection of protease activity on IgG (fig. 4) and IgM

No protease action on IgM could be demonstrated, but IgG was cleaved into two fragments (46 and 40 kDa) by the preparation from strain PMR 25, and one fragment was visible (40 kDa) with the preparation from strain PMR 40. Strain PMR 12 had no effect.

Discussion

This study showed that some *P. multocida* strains, freshly isolated from human specimens, synthesised enzymes that cleaved IgA of either subclass and IgG.

Fig. 4. Immunoblot of IgG. Lanes 1, 3, IgG control; IgG after incubation with preparation from strains PMR 25 (lane 2) and PMR 40 (4).

The failure of earlier attempts to demonstrate protease activity may have been because, as shown by our work, not all strains produce an IgA protease, or because, as has already been reported,⁷ protease production may disappear on repeated subculture. It is worth noting that strains PMR 25 and PMR 40 degrade the various substrata little if at all after more than five subcultures (data not shown).

Cleavage products were found with preparations from two freshly isolated *P. multocida* strains (PMR 25 and PMR 40). There was a slight 2–3 kDa shifting in the α band from serum IgA after contact with the bacterial supernates. This slight shift may have resulted from the cleavage of a small undetectable peptide, or from glycosidase activity. Glycosidases have already been suspected of acting concomitantly with *S. pneumoniae* IgA protease.³

A further major observation was that among the detectable fragments, two main ones were obtained from the different forms of IgA. These two fragments had apparent M_r of 36 and 44 kDa. The variations in size of these two cleavage products may have been due to variations in IgA glycosylation. With the myeloma IgAs tested, which had α heavy chains of different M_r , protease cleavage corresponded to the excision of the unseen peptides with the same M_r : 16 and 23 kDa for IgA1, 18 and 24 kDa for IgA2m(2). Whichever IgA was used, the sum of the M_r of the visible fragments exceeded the M_r of the intact α heavy chain. The sizes of these fragments were different from those produced by the classic IgA proteases, indicating cleavage that occurred outside the hinge region. This may have been

an example of sequential degradation (trypsinic type) of small peptides that were not detectable in our system, resulting mainly in two more or less stable fragments of approximately 44 and 36 kDa. The intermediate fragments obtained during the cleavage of serum IgA by protease of strains PMR 25 and PMR 40 (51 and 49 kDa respectively) support this hypothesis, as does the absence of the lightest fragment after treatment with the protease preparation from strain PMR 25. To verify this hypothesis, the kinetics of enzymatic digestion should be studied, to determine whether these fragments appear in a decreasing order of size.

The specificity of these proteases was not restricted to IgA, but extended to IgG, where again two fragments (one fragment visible for the PMR 40 protease) were present whose M_r exceeded that of the γ heavy chain. Other fragments were not demonstrated and IgM seemed to be insensitive to protease activity.

Such results may be related to those of Loomes and Senior^{9,22} who reported that *Pr. mirabilis* produced a proteolytic enzyme capable of cleaving both IgA subclasses and also IgG. The heavy chain of serum IgA1 was cleaved by this enzyme, which yielded a major fragment of about 47 kDa and a minor fragment of about 34 kDa. We intend to purify the *P. multocida* protease and determine precisely its cleavage site.

The possible role of this enzyme in the chronic carriage and virulence of *P. multocida* has yet to be defined, but clearly, the wide range of activity of the enzyme on different immunoglobulin types may be to the advantage of the organism.

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